

Exhibit C

# Expression of Integrins and Examination of Their Adhesive Function in Normal and Leukemic Hematopoietic Cells

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Adhesion of hematopoietic progenitor cells to marrow-derived adherent cells has been noted for erythroid, myeloid, and lymphoid precursors. In this report, we have characterized very late antigen (VLA) integrin expression on normal CD34<sup>+</sup> marrow progenitors, on leukemic cell lines, and on blasts from patients with acute myelogenous or monocytic leukemias. CD34<sup>+</sup> progenitor cells expressed the integrin  $\beta_1$  chain (CD29), VLA-4 $\alpha$  (CD49d), and VLA-5 $\alpha$  (CD49e). The myeloid lines KG1 and KG1a also expressed CD49d and CD49e as did the Mo7e megakaryoblastic line. CD29, CD18, and CD11a were also present on each of these cell lines. Only the Mo7e line expressed the cytoadhesins GPIIb/IIIa or GPIb. Binding of KG1a to marrow stroma was partially inhibited by antibodies to CD49d and its ligand, vascular cell adhesion molecule (VCAM-1). The majority of leukemic blasts studied expressed CD49d and CD49e as

well. Blasts from patients with acute myelomonocytic leukemia consistently bound to stroma at levels greater than 20%, and adhesion to stroma could in some cases be partly inhibited by anti-CD49d. No role for glycosylphosphatidylinositol (GPI)-linked structures was demonstrated in these binding assays because the adhesion of leukemic blasts to stroma was not diminished after treatment with phosphatidylinositol-specific phospholipase C (PI-PLC). These studies indicate that CD34<sup>+</sup> myeloid progenitors, myeloid leukemic cell lines, and leukemic blasts possess a similar array of VLA integrins. Their functional importance individually or in combination with other mediators of attachment in adhesion, transendothelial migration, and differentiation has yet to be fully elucidated.

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**N**ORMAL HEMATOPOIESIS in adult humans occurs within the bone marrow (BM) under influences of the marrow microenvironment.<sup>1</sup> These influences, which include cellular,<sup>2</sup> cytokine,<sup>3,4</sup> and extracellular matrix interactions,<sup>5</sup> all contribute to an environment that allows the hematopoietic progenitor cells to proliferate and differentiate normally. In long-term culture of the BM, an in vitro system simulating the in vivo microenvironment, an adherent stromal layer consisting of fibroblasts, macrophages, endothelial cells, and extracellular matrix is important for sustaining hematopoiesis.<sup>6</sup> Early progenitor cells are preferentially associated with the stromal layer,<sup>7,8</sup> and adhesion of early progenitor cells to marrow-derived adherent cells has been previously documented for erythroid,<sup>9</sup> myeloid,<sup>10,11</sup> and lymphoid<sup>12,13</sup> precursors. Cell-cell and cell-matrix interactions between the BM microenvironment and hematopoietic progenitors likely play an important role in the proliferation and maturation required for normal hematopoiesis.

A number of potential cell adhesion molecules, which may mediate essential cell-cell or cell-matrix interactions, have been identified in experimental models of BM.<sup>14-18</sup> The integrin superfamily of cell adhesion molecules plays a key role

in development, immune response, platelet aggregation, and tissue repair.<sup>19,21</sup> and it is currently the focus of intense study regarding its potential role in myeloid<sup>17</sup> and lymphoid hematopoiesis.<sup>12,15</sup> Integrins are heterodimer glycoproteins consisting of noncovalently linked  $\alpha$  and  $\beta$  chains. They are classified according to their  $\beta$  chain into the very late antigen (VLA) integrins ( $\beta_1$ ), leukocyte integrins ( $\beta_2$ ), cytoadhesin integrins ( $\beta_3$ ), and additional molecules expressing  $\beta_4$ ,  $\beta_5$ ,  $\beta_6$ ,  $\beta_7$  ( $\beta_p$ ), or  $\beta_8$  chains. The  $\beta_1$ (VLA) subfamily consists primarily of receptors for cell matrix components and is expressed in both hematopoietic and nonhematopoietic cells.<sup>20,22</sup> The seven members of the VLA subfamily are distinguished by the association of different  $\alpha$ -chains with the common integrin  $\beta_1$ -chain.<sup>19,21-23</sup> Hematopoietic expression of the VLA integrins includes VLA-1, VLA-2, and VLA-6 (CD49a, b, e) presence on T cells,<sup>23-25</sup> and VLA-2 and VLA-6 expression on platelets.<sup>17,25</sup> VLA-4 (CD49d) and VLA-5 (CD49e) are more widely expressed within the hematopoietic system.<sup>12,14,16,17,25</sup>

We have previously investigated the interaction of myeloid progenitors with BM stroma in vitro and reported that a subset of human marrow cells that possesses the CD34 antigen binds to marrow stromal monolayers<sup>10</sup> and, to a lesser extent, to extracellular matrices of marrow and to fibronectin and laminin.<sup>11</sup> We also reported that both normal CD34<sup>+</sup> myeloid progenitors and myeloblastic cell lines (KG1 and KG1a) express the  $\beta_2$  integrin (CD18), but the adhesive interactions between these myeloid progenitors and marrow stromal layers were not inhibited by anti-CD18 monoclonal antibodies (MoAbs) with functional blocking properties.<sup>11</sup> In this report, we examine further the expression of integrins on normal CD34<sup>+</sup> myeloid progenitors as well as on the leukemic cell lines KG1, KG1a, and Mo7e and myeloblasts obtained from leukemic patients. We also investigate the possible involvement of the VLA integrins in adhesive interactions between myeloid precursors and the marrow microenvironment through studies of binding inhibition.

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# MATERIALS AND METHODS

## Separation and Culture

**Marrow CD34<sup>+</sup> myeloid precursors.** A sterile two-step flow cytometric technique used to isolate CD34<sup>+</sup> marrow cells has been described previously.<sup>10</sup> BM aspirates were obtained from normal volunteer donors in accordance with institutional guidelines of the Research Subjects Review Board of the University of Rochester.  $5 \times 10^7$ -light-density marrow cells per sample were stained with a 1:20 dilution of fluorescein isothiocyanate (FITC)-conjugated HPCA-1, an antibody that recognizes the CD34 antigen (Becton Dickinson, Mountain View, CA), and with phycoerythrin (PE)-conjugated anti-CD10 (CALLA antigen; Coulter, Hialeah, FL). One to 5% of cells were positive for CD34 and negative for CD10. Cells were then sorted prospectively using an EPICS C flow cytometer (Coulter). An initial sort performed at high speed resulted in a population that was 60% to 70% CD34<sup>+</sup>. A second slow-speed sort resulted in a 96% to 99% pure CD34<sup>+</sup>CD10<sup>-</sup> population. These cells, which were 95% viable at the conclusion of the second sort, constituted the purified CD34<sup>+</sup> myeloid precursors subsequently used in binding assays and indirect immunofluorescence phenotyping studies. At the completion of the sort, centrifugation was performed to remove sorting sheath fluid, and the cells were resuspended in RPMI medium with 20% fetal bovine serum (FBS) for overnight storage at 37°C if further studies could not be conducted soon after sorting conclusion.

In certain experiments, CD34<sup>+</sup> cells were isolated by an avidin-biotin column adsorption technique (CellPro, Inc. Bothell, WA).<sup>26</sup>

**Cell lines.** The leukemic cell lines KG1, KG1a, and Mo7e were obtained from Dr D.W. Golde (Memorial Sloan Kettering, Rye, NY). KG1 and KG1a were maintained in RPMI-1640 (GIBCO, Grand Island, NY) culture medium with 10% FBS (Hyclone, Logan, UT). Mo7e is an interleukin-3 (IL-3)-dependent cell line and was maintained in Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO) with 20% FBS and 50 pmol/L IL-3 (kindly provided by Dr S. Clark, Genetics Institute, Cambridge, MA).

**Leukemic patient samples.** Marrow or blood obtained with informed consent was layered over Ficoll-Hypaque (specific gravity 1.077; Pharmacia, Piscataway, NJ). Light-density interface cells were washed twice and resuspended in RPMI with 10% FBS. Samples were used in binding assays only if they had greater than 80% blasts.

**Stromal cell layers.** Marrow stromal cell layers were established from BM aspirates from normal volunteers and were cultured and passaged as previously described.<sup>10</sup> Briefly, light-density BM cells were cultured in McCoy's 5A culture medium (GIBCO) supplemented with 12.5% FBS, 12.5% horse serum (Hyclone), and 1  $\mu$ mol/L hydrocortisone (Sigma Chemical Co. St. Louis, MO). These adherent cell layers were used in binding assays only after passage of more than three times. At final passage, the adherent layers were seeded into either Falcon 24-well plates (Becton Dickinson, Lincoln Park, NJ) for cell line chromium-binding studies or into 35-mm tissue culture plates (Corning, Corning, NY) for CD34<sup>+</sup> progenitor cell adhesion assays. The adherent layers were allowed 5 to 7 days of incubation after the final passage to ensure adequate confluent matrix formation for cell-binding assays.

## Antibodies and Reagents

Antibodies and additional reagents were obtained from Becton Dickinson Immunocytometry Systems, Mountain View, CA: (HPCA-1 [CD34, IgG1] and FITC-conjugated goat antimouse isotype control [IgG1], Coulter Immunology, Hialeah, FL; (CD29 [VLA  $\beta$  chain, IgG1], CD10PE, and goat antimouse isotype controls for IgG1, IgG2a, and IgG3), Fisher Scientific, Orangeburg, NJ; (FITC-conjugated isotype specific goat antimouse IgG1, PE-conjugated isotype specific goat antimouse IgG2b and IgG3), Telios Pharmaceuticals, San Diego,

CA; (CD49b [VLA-2  $\alpha$  chain, IgG1], CD49c [VLA-3  $\alpha$  chain, IgG1], and CD49e [VLA-5  $\alpha$  chain, IgG3]), AMAC, Westbrook, ME; (CD49d [VLA-4  $\alpha$  chain, IgG1], CD49f [VLA-6  $\alpha$  chain, IgG2a], CD61 [GPIIIa or  $\alpha_3$ , IgG1], and CD34 FITC conjugate), Tago, Burlingame, CA; (FITC-conjugated F(ab')<sub>2</sub> goat anti-mouse Ig and PE-conjugated F(ab')<sub>2</sub> goat anti-mouse Ig), Antibodies L1 (anti-LFA-1  $\alpha$  chain, CD11a), 44 alpha (anti-Mol $\alpha$ -chain, CD11b), L29 (anti-p150.95  $\alpha$ -chain, CD11c), and 10F12 (anti- $\beta_2$ -chain, CD18) were gifts of Dr A. Armaout (Massachusetts General Hospital, Boston, MA). Antibody 12.8, an IgM molecule recognizing CD34, was a gift of Dr R. Berenson, Seattle, WA. Anti-VCAM (4B9, IgG1) was a gift from Dr J. Harlan (Harborview Medical Center, Seattle, WA). For diagnostic immunophenotyping of leukemic blasts, anti-CD14, anti-CD19, anti-CD33, and anti-CD34 were obtained from Coulter. Antibodies AP1 (anti-GPIb), AP2 (anti-GPIIb/IIIa), AP4 (anti-GPIIb), and AP5 (anti-GPIIa) were gifts of Dr T.J. Kunicki (The Blood Center of Southeastern Wisconsin, Milwaukee). Phosphatidylinositol-specific phospholipase C (PI-PLC) was purchased from ICN Biomedicals (Costa Mesa, CA). Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 were gifts of Dr S. Clark.

## Adhesion Protein Expression

Cell lines were stained for indirect immunofluorescence by incubating with antibody to the adhesion protein at 4°C for 30 minutes followed by three washes in PBS. Cells were then incubated at 4°C for 30 minutes with FITC-conjugated goat anti-mouse Ig. After the final wash, cells were resuspended in 1% paraformaldehyde in PBS at 4°C for flow cytometry analysis. Stained cells were analyzed on an EPICS C flow cytometer. The mean log green fluorescence channel of the cell lines was determined directly from a single parameter histogram. The mean log fluorescence channel was converted to linear equivalents as previously described.<sup>27</sup> In order to estimate the fluorescence intensity specifically due to the test adhesion protein antibody, the mean fluorescence intensity of cells stained with isotype-specific control antibody was subtracted from that of cells stained with the adhesion protein antibody to obtain the final mean fluorescence intensity measurement. To ensure that only specific receptor expression was evaluated, only fluorescence measurements  $\geq 5$  were defined as positive after subtraction of background staining.

For phenotyping of adhesion proteins on normal CD34<sup>+</sup> myeloid progenitors, two-color immunofluorescence was used with light-density marrow cells stained with an adhesion marker versus CD34.  $2 \times 10^6$ -light-density bone marrow cells were incubated with 12.8 anti-CD34 and unconjugated test antibody for 30 minutes at 4°C in PBS plus 20% human AB serum and then washed twice with cold PBS. The cells were then incubated with FITC-conjugated goat anti-mouse IgG specific for the isotype of the unconjugated "test" antibody used and with goat antimouse IgM PE. After staining, cells were resuspended in 1% paraformaldehyde in PBS and kept at 4°C for flow cytometry analysis. Stained cells were analyzed on an EPICS Profile flow cytometer (Coulter) using standard light scatter gates for mononuclear cells to obtain a two-parameter histogram of log green fluorescence (FITC) versus log red fluorescence (PE).

## Binding Assays

**Chromium binding assay.** Binding experiments with cell lines were performed as previously described<sup>22</sup> with minor modifications. KG1, KG1a, or Mo7e cells ( $5 \times 10^6$ ) were labeled with 100  $\mu$ Ci <sup>51</sup>Cr (Amersham, Arlington Heights, IL) for 30 minutes at room temperature. Cells were then washed three times in more than 10 vol of PBS. Cells were then resuspended in RPMI-1640 medium with 10% FBS at a concentration of  $1.0 \times 10^6$  cells/mL. One-half milliliter ( $5 \times 10^5$  cells) were plated over the stromal layer or plastic-coated 24-well tissue culture plate for 2 hours at 37°C. The medium and non-

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with the degree of binding to stroma nor did possession of CD14, a monocytic marker, correlate with degree of binding to tissue culture plastic. In five cases, the effect of PI-PLC on leukemic blast binding to stroma was investigated. Cells were treated with 120 mU PI-PLC for 30 minutes at 37°C, washed once, and plated in the previously described adherence assay. As shown in Fig 3, in only two cases did mean binding decrease in presence of PI-PLC: by 21% in sample 3 and by 27% in sample 5.

#### *Inhibition of Adhesion by Blocking Antibodies*

To study which of the expressed adhesion proteins might be involved with adhesion of myeloid progenitors to the BM stroma, we used an *in vitro* model of stromal adhesion to conduct inhibition studies with blocking antibodies to the integrins of interest.

Each of the adhesion receptor antibodies used in the inhibition experiments blocks adhesion at the concentration used. CD29 and CD49d antibodies inhibited adhesion of a B-cell line, NALM-6, to BM fibroblasts by 57% and 52%, respectively.<sup>12</sup> The CD49e and anti-VCAM antibodies were used at a concentration previously demonstrated to show blocking of VLA-5-dependent and VCAM-dependent adhesion, respectively.

**CD34<sup>+</sup> cell lines and leukemic myeloblasts.** The effect of various blocking antibodies on adhesion of the myeloblastic cell lines KG1, KG1a, and Mo7e to bone marrow stroma is shown in Fig 4. Binding of the KG1a, KG1, and Mo7e cells to normal bone marrow stroma was not significantly decreased by anti-CD49e (anti-VLA-5 $\alpha$ ) or CD29 (anti- $\beta_1$ ). Anti-CD49d (anti-VLA-4 $\alpha$ ), and anti-VCAM had no effect on the adhesion of KG1 or Mo7e to marrow stromal layers. As shown in the case of Mo7e, the combination of anti-VLA-4 $\alpha$  and anti-CD29 did not block adhesion either. In contrast, KG1a binding to marrow stroma was partially inhibited by both anti-VLA-4 $\alpha$  and anti-VCAM ( $P < .05$  by paired *t*-test) (Fig 4A). The combination of these two anti-

bodies did not result in greater inhibition than either used alone. No difference in the degree of inhibition was observed between addition of the test antibody at the time of target cell incubation over stromal layers versus preincubation of antibody with ligand-containing cells for 30 minutes at 4°C.

In three cases of myeloid leukemia where sufficient blasts were obtained to study effects of antibodies to VLA integrins on stromal adhesion, anti-VLA-4 $\alpha$  inhibited blast binding by a mean of  $18\% \pm 4.9\%$  (range 10% to 27%). In two cases studied, anti-VLA-5 $\alpha$  did not result in significant inhibition of blast binding.

**Normal CD34<sup>+</sup> progenitors.** We have previously shown that normal CD34<sup>+</sup> progenitors attach to stromal layers using a binding assay in which the readout is numbers of CFU-GM colonies.<sup>10</sup> Using this same assay, inhibition of adhesion of normal CD34<sup>+</sup> progenitors to BM stroma was examined. When the binding assay was performed in the presence of anti-CD29 ( $\beta_1$ ) (1:100 dilution of 4B4 antibody), inhibition of adhesion of normal progenitors to marrow stroma was 53% ( $\pm 4.2\%$ ,  $n = 3$ ). Data from these three experiments are shown in Fig 5A. Addition of anti-VLA-4 $\alpha$  chain antibody did not enhance the inhibition seen with anti- $\beta_1$  alone.

In addition, to assess effects of these blocking antibodies upon the entire population of CD34<sup>+</sup> cells, <sup>51</sup>Cr labeling of column-adsorbed CD34<sup>+</sup> cells was performed followed by the standard adhesion assay. As shown in Fig 5B, anti-CD29 (VLA- $\beta_1$  chain) gave significant binding inhibition in this population as well ( $P < .01$  by paired *t*-testing), whereas anti-VLA-4 $\alpha$ , anti-VCAM, and anti-VLA-5 $\alpha$  did not.

**Effects of cytokines on binding of leukemic cell lines.** To assess whether incubation of progenitor or leukemic cells with growth factors would enhance adhesion as has been previously reported for transplanted marrow in murine models,<sup>28</sup> or to examine whether exposure of the stromal layers to inflammatory/immune modulators would influence adhesion, adhesion assays were performed after incubation with appropriate cytokines. As shown in Table 4, neither IL-3 nor

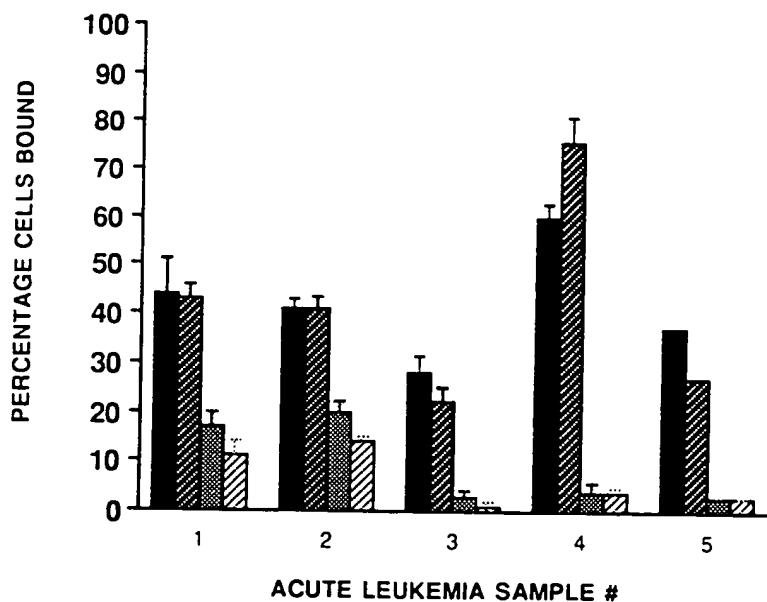


Fig 3. Shown is the percentage of myeloblasts bound to stromal or plastic layers in the presence or absence of PI-PLC treatment in five cases of acute myeloblastic leukemia (AML). Similar treatment of blood monocytes with PI-PLC decreased CD14 antigen density by greater than 50%.  $N = 3$  except for sample No. 5 performed in duplicate. (■), Stroma - PLC; (▨), stroma + PLC; (□), plastic - PLC; (▤), plastic + PLC.

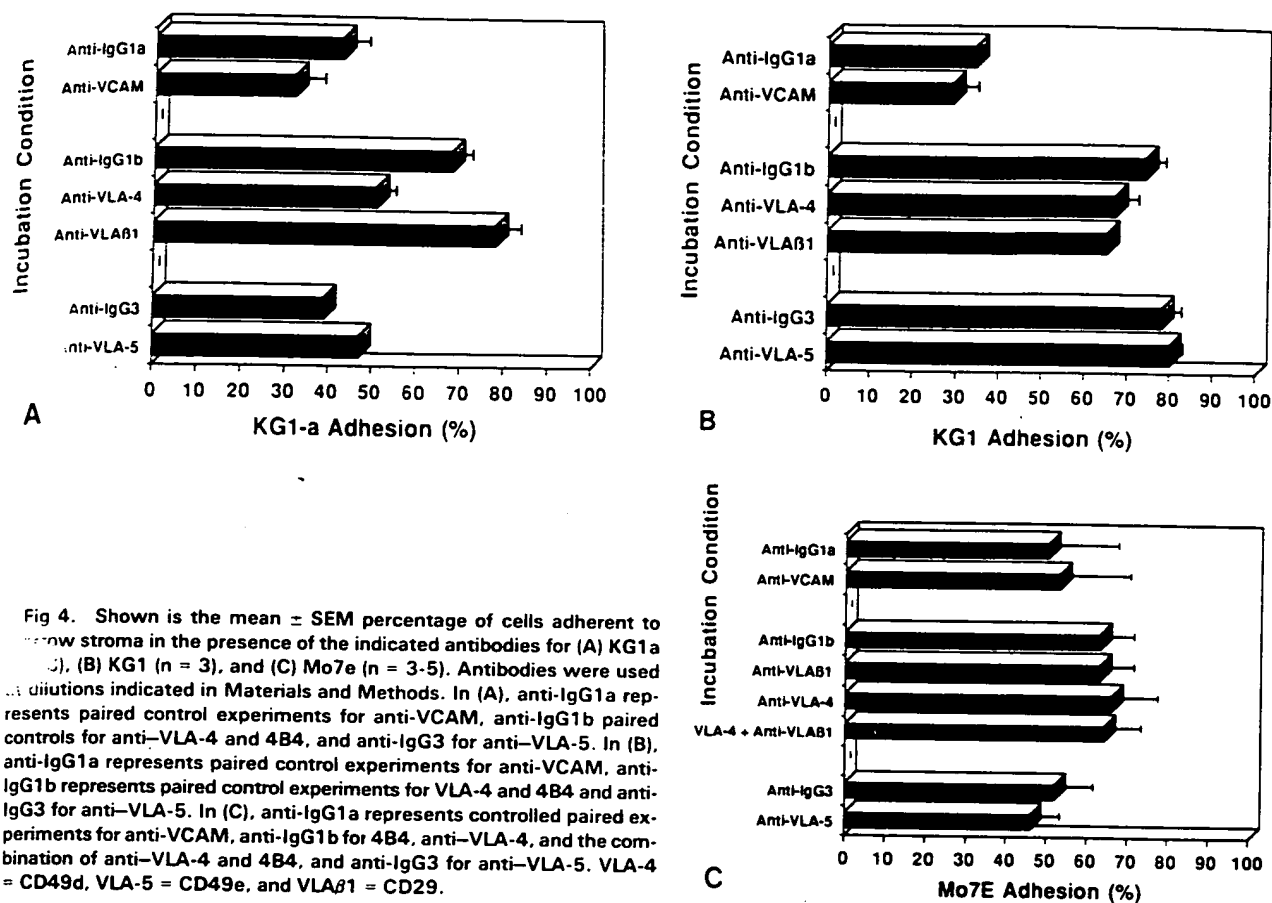


Fig 4. Shown is the mean  $\pm$  SEM percentage of cells adherent to marrow stroma in the presence of the indicated antibodies for (A) KG1a (n = 3), (B) KG1 (n = 3), and (C) Mo7e (n = 3-5). Antibodies were used at dilutions indicated in Materials and Methods. In (A), anti-IgG1a represents paired control experiments for anti-VCAM, anti-IgG1b paired controls for anti-VLA-4 and 4B4, and anti-IgG3 for anti-VLA-5. In (B), anti-IgG1a represents paired control experiments for anti-VCAM, anti-IgG1b represents paired control experiments for VLA-4 and 4B4 and anti-IgG3 for anti-VLA-5. In (C), anti-IgG1a represents controlled paired experiments for anti-VCAM, anti-IgG1b for 4B4, anti-VLA-4, and the combination of anti-VLA-4 and 4B4, and anti-IgG3 for anti-VLA-5. VLA-4 = CD49d, VLA-5 = CD49e, and VLAβ1 = CD29.

BM-CSF at concentrations of 50 U/mL and 100 U/mL, respectively, affected binding of KG1a or KG1 to marrow stromal layers. Also, pretreatment of stromal layers with  $10^{-8}$  mol/L PMA (phorbol myristate acetate) or 10 U/mL IL-1α did not alter the degree of adhesion of the KG1a or Mo7e cell lines nor of normal CD34<sup>+</sup> myeloid progenitors to such layers (Table 5).

#### DISCUSSION

The β<sub>1</sub> (VLA) integrins are generally associated with adhesion to extracellular matrix components. In this study, it has been documented that early myeloid cells, including normal CD34<sup>+</sup> progenitors, early leukemic cell lines, and acute leukemia myeloblasts all possess a similar array of VLA integrins. Most prominently expressed are CD29 (the common β<sub>1</sub> chain; CD49e (VLA-5α), a receptor for the RGDs cell-binding site of fibronectin; and CD49d (VLA-4α), which can bind to fibronectin domains<sup>29,30</sup> as well as to a cell-associated ligand, VCAM-1.<sup>31</sup> CD49b and CD49c were minimally expressed on all of these cell types. VLA-6, a receptor for laminin, was present only on established leukemic cell lines, and most prominently on Mo7e, a line with some megakaryocytic properties. We have shown previously that normal CD34<sup>+</sup> myeloid progenitors possess CD18,<sup>11</sup> and here it is shown that immature cell lines (CD34<sup>+</sup>) also possess CD18 and CD11a. No significant expression of cytoadhesins was found on the three cell lines tested except on Mo7e, which

has megakaryocytic properties. Normal CD34<sup>+</sup> progenitors did not possess the β<sub>3</sub> chain (CD61, vitronectin β receptor/gpIIb).<sup>32</sup>

The spectrum of β-integrin antigen expression documented here is in keeping with that noted by others using different assay methods. Saeland et al<sup>33</sup> have reported a similar array of integrin antigen expression on CD34<sup>+</sup> cells from marrow and cord blood, and a repertoire of integrin expression during erythroid maturation has also been described.<sup>34</sup> Soligo et al.<sup>17</sup> using an immunohistochemical detection method, also found VLA-4 presence on immature hematopoietic cells and restriction of β<sub>3</sub> cytoadhesin molecules to megakaryocytes and platelets when unfractionated BM was studied. They also noted only a minor role for β<sub>2</sub> integrins in early hematopoiesis and found variability of β<sub>3</sub> integrin expression on acute myelogenous leukemia specimens, whereas VLA-2, VLA-3, and VLA-6 were absent on nonlymphoid progenitors.<sup>17</sup>

In the study presented here, VLA-5α (CD49e) was seen on all early myeloid cell types (normal CD34<sup>+</sup> progenitors, leukemic cell lines, and acute leukemia myeloblasts) with a relative increase seen in some cases of acute myelocytic leukemia (AML), probably reflecting the cell-maturation spectrum involved in leukemogenesis.<sup>17</sup> In addition to VLA-5α and the common β<sub>1</sub> chain (CD29), only VLA-4α (CD49d) was prominently expressed on normal early myeloid cells. Within the acute leukemia samples studied, VLA-4α expression did not seem to correlate with maturation as even an

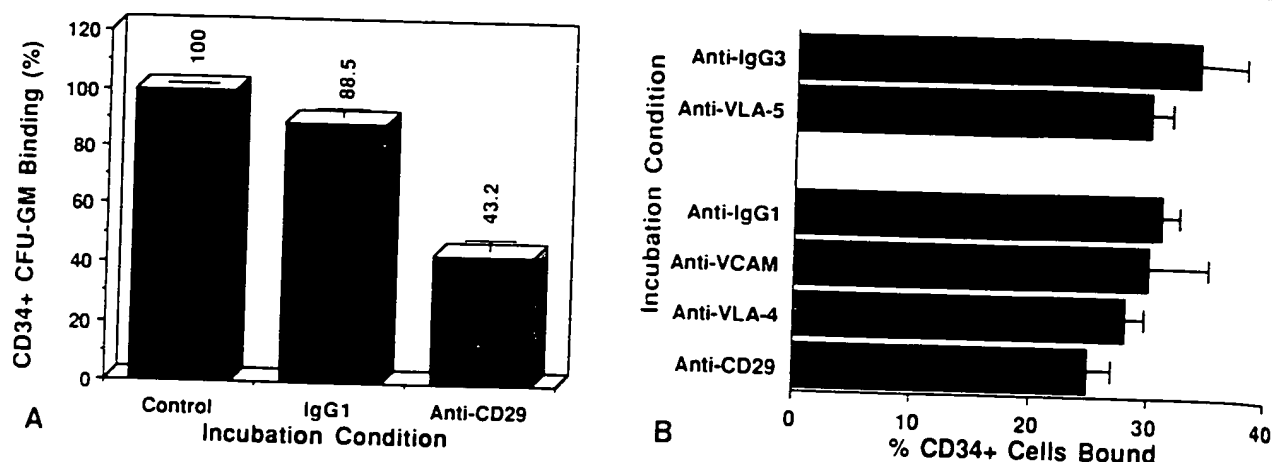


Fig 5. (A) Shown are the mean  $\pm$  SEM percentage of CFU-GM bound to marrow stroma in the presence of anti-CD29 (anti- $\beta_1$ ) or in the presence of an IgG1 control antibody as compared to binding to stroma without antibody present (100%);  $n = 3$  for all conditions. (B) The percentage of CD34<sup>+</sup> cells bound to marrow stroma in the presence of the indicated antibodies is shown with all data obtained in a parallel fashion with the appropriate isotype specific control antibody;  $n = 4$  for all IgG1-paired conditions and  $n = 3$  for VLA-5/IgG3. VLA-4 = CD49d and VLA-5 = CD49e.

M3 specimen with no CD34<sup>+</sup> blasts expressed significant levels of VLA-4 $\alpha$ . VLA-4 has been found on cultured T lymphocytes to function as a receptor for the heparin-II and IIICS domains of fibronectin.<sup>29</sup> VLA-4 $\alpha$  has also been found to be a ligand for VCAM-1, an adhesion molecule of the immunoglobulin gene superfamily found on endothelial cells stimulated with IL-1 $\alpha$ , lipopolysaccharide, or tumor necrosis factor  $\alpha$ . It is also constitutively expressed on dendritic cells, renal tubular epithelial cells, and tissue macrophages.<sup>31</sup> VCAM has also been found on marrow stromal cell layers such as those used here.<sup>35</sup>

To what extent CD49d (VLA-4 $\alpha$ ) or CD49e (VLA-5 $\alpha$ ) antigens participate in homing phenomena or in attachment of myeloid progenitors to marrow microenvironmental components remains unknown. The data presented here show no role for VLA-5 $\alpha$  in adhesion of CD34<sup>+</sup> cells to stroma as measured in a specific adhesion assay. This is in keeping with previous studies that show no role for RGD sequences in such adhesion processes.<sup>36,37</sup> In contrast, RGD sequences have

been found to have a role in adhesion of erythroid progenitors to marrow.<sup>33</sup>

Antibody inhibition data shown here suggest a role for CD29 ( $\beta_1$  integrin) in adherence of CD34<sup>+</sup> CFU-GM-forming cells to marrow stroma and participation of VLA-4 $\alpha$  (CD49d) in adhesion of some myeloid progenitors (KG1a cell line and three samples from leukemia patients) to stroma. Data shown here with the KG1a cell line would suggest that VCAM-1 on human marrow stromal cells may participate in leukemic blast adhesion as the ligand for VLA-4. Such an interaction has also been noted for binding of lymphocytes or lymphocyte progenitors to endothelial cells,<sup>38,39</sup> to murine marrow stromal cells,<sup>13,40</sup> or to human marrow fibroblasts expressing VCAM-1,<sup>12</sup> and, more recently, for adhesion of myeloid and erythroid progenitors to marrow stromal cells.<sup>35</sup>

In no instance did antibodies to VCAM-1, VLA-4 $\alpha$  (CD49d),  $\beta_1$  (CD29), or other VLA integrins completely inhibit myeloid progenitor adhesion to marrow stroma, and, in most cases, inhibition of adhesion noted was minimal. This might suggest a role for other classes of integrins or other types of adhesion receptors in the interaction of leu-

Table 4. Effect of GM-CSF and IL-3 on GK1a and KG1 Adhesion

	KG1a	KG1a - GM-CSF	KG1a	KG1a + IL-3
Stroma	65 $\pm$ 3.0	63 $\pm$ 6.1	63 $\pm$ 11	68 $\pm$ 9.0
Plastic	1.3 $\pm$ 0.6	2.0 $\pm$ 1.0	3.8 $\pm$ 1.2	4.0 $\pm$ 0.7

	KG1	KG1 - GM-CSF	KG1	KG1 + IL-3
Stroma	68 $\pm$ 2.9	74 $\pm$ 3.5	18 $\pm$ 5	19 $\pm$ 6
Plastic	2.7 $\pm$ 0.6	3.7 $\pm$ 1.2	ND	ND

Data represent the mean  $\pm$  standard deviation percentage of the indicated cell line which adhered to marrow stromal layers or plastic in the presence or absence of GM-CSF (100 U/mL) or IL-3 (50 U/mL). Experiments were performed in a paired fashion with  $n = 3$  for all conditions except for KG1a + IL-3 experiments and their controls for which  $n = 5$ . Abbreviation: ND = not done.

Table 5. Effect of IL-1 $\alpha$  and Phorbol Myristate Acetate (PMA) on Cell Adhesion

	CD34 <sup>+</sup> Progenitors	KG1a	Mo7e
Stroma	14 $\pm$ 3.2 ( $n = 3$ )	23 $\pm$ 9.5 ( $n = 4$ )	43 $\pm$ 9 ( $n = 3$ )
Stroma + IL-1 $\alpha$	17 $\pm$ 3.5 ( $n = 3$ )	26 $\pm$ 8.6 ( $n = 4$ )	49 $\pm$ 12 ( $n = 3$ )
Stroma	38 $\pm$ .89 ( $n = 3$ )	41 $\pm$ 4.7 ( $n = 3$ )	48 ( $n = 2$ )
Stroma + PMA	41 $\pm$ 2.3 ( $n = 3$ )	42 $\pm$ 4.7 ( $n = 3$ )	53 ( $n = 2$ )

Data shown are mean  $\pm$  SEM percentage of the indicated cell type bound to stromal layers grown in the presence or absence of 10 U/mL IL-1 $\alpha$  or  $10^{-8}$  mol/L PMA for 24 hours. Experiments were performed in a paired fashion,  $n =$  number of experiments.

kemia cells with marrow. These might include heparin-binding receptors, proteoglycans, L-selectin and its mucin-like ligand.<sup>33,41</sup> CD44-hyaluronate receptors,<sup>42</sup> hemonectin,<sup>43</sup> thrombospondin,<sup>44</sup> other collagen receptors,<sup>37</sup> or other fibronectin receptors.<sup>45</sup> A role for the 33/66-Kd carboxy-terminal heparin-binding cell adhesion domains of fibronectin in interactions between primitive marrow progenitors and intact irradiated marrow stroma has been found.<sup>46</sup> This interaction appeared to involve the FN-C/H II site. Also, other anchored factors such as membrane-bound basic fibroblast growth factor,<sup>47</sup> macrophage colony-stimulating factor (M-CSF, CSF-1),<sup>48</sup> or *c-kit* ligand (stem cell factor)<sup>49</sup> may participate in this adhesion process. Figure 6 illustrates schematically some of the possible receptor-ligand interactions that might contribute to precursor (CD34<sup>+</sup>) attachment to marrow stroma.

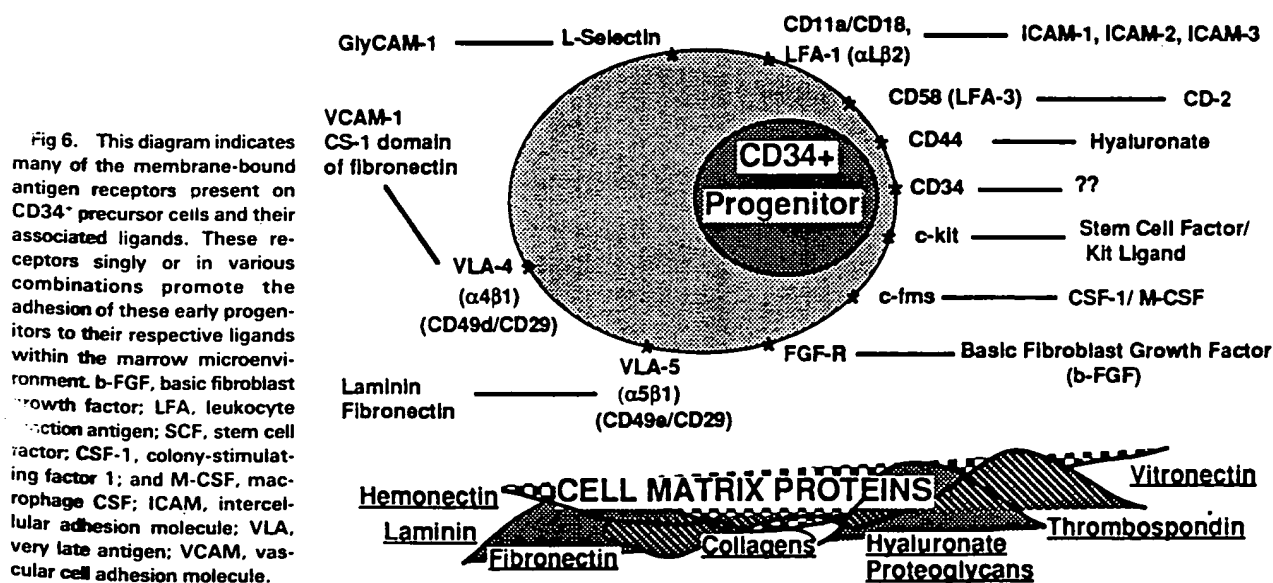
The lack of inhibition seen with anti-CD29 in the case of myeloid/megakaryoblastic cell lines does not necessarily rule out a role for  $\beta_1$  integrins in their adhesion to stroma. Changes in conformation of some integrin receptors after antibody binding can "activate" or alter the affinity of ligand recognition. This has been reported for another monoclonal antibody to CD29 (8A2) that stimulated the binding of U937 cells to CHO cells transfected with VCAM-1 complementary DNA (cDNA)<sup>50</sup> and for glycoprotein IIb/IIIa,  $\alpha_{IIb}\beta_3$ .<sup>51</sup> Whether such a phenomenon might be operational in the adhesion of myeloid leukemic progenitors to stroma can only be speculated on. It is also possible that to detect the role of the CD49d(VLA-4 $\alpha$ )/VCAM complex in mediating progenitor adhesion to the microenvironment, one may have to maximize the stromal expression of VCAM-1 by cytokine combinations, as recently shown by Simmons et al.<sup>35</sup>

In this work, the presence of a similar array of VLA integrins on freshly isolated leukemia blasts as on tissue culture-adapted cell lines and normal CD34<sup>+</sup> progenitors was identified. Leukemic marrow blasts demonstrated, on average, a

comparable degree of binding to marrow stromal layers as did these other cell types. Although the number of samples reported upon here is small, the presence of CD49d and CD49e was consistently noted, and the degree of blast adhesion to stromal layers was independent of subtype: ie. myelogenous versus monocytic versus promyelocytic. In those cases where adhesion inhibition was studied with antibody to CD49d, a small degree of inhibition was noted.

This ability of leukemic blasts to adhere to marrow stromal layers is surprising given the common presence of blood blasts in these cases even when marrow cellularity does not suggest a crowding effect. Because VLA integrin expression appears similar to that of normal progenitors, it is possible that blast egress occurs because of altered function or affinity of these receptors for their respective ligands. It is also possible that these blasts lack adhesive receptors of other uncharacterized classes as has been described for progenitors in chronic myelogenous leukemia (CML).<sup>52</sup> Unlike the situation in CML, the acute myelo(monocytic) blast adhesion to stroma was not consistently PI-PLC linked.<sup>53</sup> Another explanation for blast exit from the marrow would be altered receptor affinity or changes in integrin phenotype on egress. Such possibilities could be explored by comparing the adhesive properties of circulating blood blasts to marrow blasts. Hematopoietic growth factors such as GM-CSF and IL-3 have been reported to influence adhesion or aggregation of mature granulocytes and monocytes,<sup>54,55</sup> but their presence did not influence binding of early myeloid cell lines to marrow stromal layers in our hands.

As cells of the granulocyte lineage mature, they lose VLA-4 $\alpha$ , whereas monocytes and eosinophils<sup>56</sup> retain this protein. Changes in integrin phenotype with blast differentiation may partially explain the propensity of monoblasts for tissue invasion. Other factors regulating acute myelogenous leukemia blast traffic may relate to their responses to chemotactic factors or their ability to elaborate collagenases<sup>46</sup> or enzymes



such as the human heparin-binding elastase homologue (CAP37, azurocidin) that mediates reversible fibroblast and endothelial cell contractility,<sup>57</sup> thereby facilitating cell egress into extravascular spaces. Further studies will be required to assess the role of the VLA integrins in leukemia blast egress from the marrow and any role that they might play in stem cell homing during transplantation.<sup>58</sup>

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